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Elevated BALF concentrations of α- and β-defensins in patients with pulmonary alveolar proteinosis

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Summary
Defensins are endogenous antibiotics and regulators of inflammation, immunity and wound repair. Their concentrations are substantially increased in bronchoalveolar lavage fluid (BALF) of patients with infectious lung diseases. α-defensin (HAD) levels are also elevated in patients with idiopathic pulmonary fibrosis (IPF) and correlated with the decline in pulmonary function tests, suggesting the association of defensins with the pathogenesis of interstitial lung diseases. The aim of this study was to determine the profile of defensins in interstitial lung diseases. Serum and BALF levels of HAD and β-defensin 1 and 2 (HBD-1, and -2) were measured by radioimmunoassay in 63 patients with interstitial lung diseases, including idiopathic pulmonary alveolar proteinosis (PAP), IPF, nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP) and pulmonary sarcoidosis, and in 9 healthy volunteers as controls. Levels of HAD in BALF of patients with PAP were significantly higher than those in controls and patients with COP and sarcoidosis. Serum levels of HAD in all groups were significantly higher than those in controls. Levels of HBD-1 and -2 in BALF of patients with PAP were extremely high in all subjects. Serum levels of HBD-1 were higher in all patient groups, with the exception of those with PAP, and those of HBD-2 were also higher in patients with IPF and sarcoidosis, compared with controls. BALF of PAP patients, but not IPF patients and controls, expressed antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Our findings suggest different kinetics of HAD and HBD-1 and -2 in serum and BALF of interstitial lung diseases and that these antimicrobial peptides in the airway lumen may contribute to prevention of bacterial airway infections in PAP.

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Introduction

The human airways are protected from invading microorganisms by a highly efficient innate immune system. Antimicrobial peptides produced by inflammatory cells and airway epithelial cells are key elements in this innate immune system.[1,2] A major subgroup of the antimicrobial peptides is the family of defensins. Defensins are endogenous antibiotics against Gram-positive and -negative bacteria, fungi and enveloped viruses, which contribute to host defense by disrupting the cytoplasmic membrane of microorganisms.[1] Recent studies indicate the importance of these peptides as effectors of innate immunity by killing microorganisms, but also as regulators of inflammation, immunity and wound repair.[1,2] In humans, six α-defensins (HAD) have been recognized to date; four forms that are exclusive to leukocytes [human neutrophil peptides (HNPs)-1 to -4] and two others found in Paneth cells in the small intestine.[1,2] Because HNPs-1, -2 and -3 constitute 5–7% of the total protein content of the human neutrophil and 30–50% of the total protein content of the azurophilic granules, it is thought that they are the most abundant antimicrobial proteins present in the neutrophil. On the other hand, β-defensin (HBD), the second class of defensin, was identified in bovine neutrophils and in epithelial cells of bovine tongue and trachea.[1] Human HBD-1 was originally isolated from blood filtrate and is expressed constitutively in epithelia of the urogenital tract, trachea and respiratory tract.[1] HBD-2, isolated from psoriatic scale extracts, is expressed mainly in human skin, trachea and lung and its expression increases in response to infections and inflammatory mediators.[1,2] HBD-1 and -2 are currently thought to contribute to antimicrobial defense in these tissues.

Previous reports have demonstrated the association between these defensins and various infectious lung diseases.[3–7] Since defensins may also cause tissue injury, modulate immunology, and contribute to repair processes,[1,2,8] the potential role of defensins in the pathogenesis of noninfectious lung diseases, including interstitial lung diseases, is also of interest. In this context, an enhanced antibacterial activity was found in bronchoalveolar lavage fluid (BALF) material from patients with sarcoidosis and this activity was present as several antibacterial components, including HAD.[9] We previously reported that concentrations of HAD in plasma of patients with idiopathic pulmonary fibrosis (IPF) were significantly higher compared with control subjects, and correlated inversely with pulmonary function tests.[10] Positive immunohistochemical staining of HAD was observed inside and outside neutrophils in dense fibrotic lung areas obtained from these patients.[10] In acute respiratory distress syndrome (ARDS), HAD levels of plasma and BALF were higher than in controls and also correlated with the Lung Injury Score.[11] These findings suggest that defensins have clinical relevance to the status of different interstitial lung diseases as well as pulmonary infectious diseases. However, studies of the role of these antimicrobial peptides, especially HBD, have not been reported in interstitial lung diseases, including idiopathic pulmonary alveolar proteinosis (PAP), which is well known for its association with susceptibility to pulmonary infections.

The aim of the present study was to determine the profile of defensins in various interstitial lung diseases. Specifically, we measured the serum and BALF concentrations of HAD, HBD-1 and -2 by radioimmunoassay (RIA). The results showed extremely high levels of these defensins in BALF of patients with PAP, with antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Materials and methods

Sample preparation

Seventy-two subjects of this study were patients and healthy volunteers enrolled in the Hospitals of Nagasaki University School of Medicine. They included 7 patients with PAP (4 males and 3 females; age 55 [42–68] years), 11 with IPF (9 males and 2 females; age 62 [47–73] years), 15 with idiopathic nonspecific interstitial pneumonia (NSIP) (8 males and 7 females; age 57 [30–76] years), 7 with cryptogenic organizing pneumonia (COP) (4 males and 3 females; age 62 [26–79] years), 23 with pulmonary sarcoidosis (7 males and 16 females; age 54 [25–78] years) and 9 healthy volunteers (5 males and 4 females; age 42 [21–66] years). None of the enrolled patients had received steroids or other immunosuppressive therapy at the time of clinical sample collection. Patients with cancer in any organ and those suspected to have malignancy were excluded from the study. The diagnosis was confirmed pathologically by surgical lung biopsy in all patients with IPF, NSIP, and COP.[12] Patients with usual interstitial pneumonia, NSIP and bronchiolitis obliterans organizing pneumonia associated with collagen vascular diseases were excluded from the study. Sarcoidosis patients were diagnosed using clinical, functional, radiographic and histological criteria as previously reported.[13] All healthy volunteers had normal chest radiographs, were free of symptoms and not taking any medications. The study protocol was approved by the Human Ethics Review Committees of Nagasaki University School of Medicine and a signed consent form was obtained from each subject.

Bronchoalveolar lavage

After informed consent was obtained, BAL was performed before treatment as previously described in Mukae et al.[10] The bronchoscope was wedged into one of the segmental or subsegmental bronchi of the right middle robe. An aliquot of 50 ml sterile saline at body temperature was instilled through the bronchoscope. The fluid was immediately retrieved by gentle suction using a sterile syringe, and the procedure was repeated three times. BALF was passed through two sheets of gauze and then centrifuged at 500*g* for 10 min at 4 °C. After washing twice with phosphate-buffered saline (PBS) free of calcium or magnesium (PBS, Gibco, UK), the remaining cells were suspended in PBS supplemented with 10% heat inactivated fetal calf serum and counted using a hemocytometer. An aliquot was then diluted to a concentration of 2×10^5 cells/ml, and 0.2 ml of cell suspension was spun down onto a glass slide at 1100 rpm for 2 min using a Cytospin 2 cytocentrifuge (Shandon Instruments, Sewickley, PA). The remaining fluid was centrifuged at 500*g* for 5 min, and supernatant was stored

at −80 °C until examined. The prepared slides were dried, fixed, and then stained using a May–Giemsa method. More than 200 cells were identified using a photomicroscope. A serum sample was also collected from each subject and stored at −80 °C on the same day.

HAD assay

The concentration of HAD was measured by RIA established in our laboratory.[14] A diluted sample or standard peptide solution (100 μl) was incubated for 24 h with 100 μl of antiserum diluent (final dilution of 1/21,000). ^{125}I-labeled HAD solution (16,000 cpm in 100 μl) was then added, and the mixture was incubated again for 24 h. In the next step, normal rabbit serum and anti-rabbit IgG goat serum were added, and the samples were stored for 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4 °C and duplicate assays were performed. The antiserum recognized HNP-1, -2, and -3 equally on a molar basis, so the RIA data were expressed as the sum of HNPs1–3 and their precursor proteins, the presence of which was confirmed by simultaneous measurements using reverse phase high-performance liquid chromatography and RIA.[15] The respective intra- and interassay coefficients of variation were 3.5% and 8% at 50% binding, respectively.

HBD assay

The concentrations of HBD-1 and 2 were also measured by RIA established in our laboratory.[16,17] Human HBD was radioiodinated by the lactoperoxidase method and the ^{125}I-labeled peptide was purified by RP-HPLC on a TSK ODS 120A column (Tosoh Co.). The incubation buffer for RIA was 50 mM sodium phosphate (pH 7.4) containing 0.25% bovine serum albumin (BSA) treated with *N*-ethylmaleimide, 80 mM NaCl, 25 mM EDTA 2Na, 0.05% NaN3, 0.1% Triton X-100, and 3.1% dextran T-40. The diluted sample or a standard peptide solution (100 μl) was incubated for 24 h with 100 μl of diluted antiserum (final dilution of 1/460,000 and 1/4,200,000, respectively). The tracer solution (16,000–18,000 cpm in 100 μl) was added and the mixture was incubated for 24 h, after which normal rabbit serum and anti-rabbit IgG goat serum were added, and the whole preparation was stored for a further 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4 °C and the samples were assayed in duplicate.

Bactericidal assay

The antimicrobial activities of each BALF against *P. aeruginosa* strain PAO-1 and *S. aureus* strain ATCC29213 were tested with colony count assay.[18] Briefly, bacteria were cultured overnight at 37 °C in Brain–Heart-Infusion (BHI, Becton Dickinson Microbiology Systems, Cockeysville, MD). An aliquot of this culture was transferred to the fresh BHI and incubated for 3 h at 37 °C to obtain cells in logarithmic-phase growth. Following the precipitation of bacteria by centrifugation at 800*g* for 10 min, samples were washed in PBS and quantified spectrophotometrically by measuring optical density at 620 nm. A culture volume containing 5×10^6 bacterial colony-forming units (CFU) was then added to 500 μl of each BALF that had been filtered with Millex®-LG 0.20-μm filter (Millipore, Cork, Ireland). The mixtures were incubated for 4, 6, and 8 h in the experiments with *P. aeruginosa*, and for 2, 4, 6, and 8 h in the experiments with *S. aureus* at 37 °C, and then serially diluted and spread on Mueller–Hinton II agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) for 18 h at 37 °C, after which the colonies were counted.

Statistical analysis

Data were expressed as median (range). Differences between groups were examined using one-way analysis of variance. The posthoc test used Fisher's PLSD test. The Student's *t*-test was used for comparisons between two groups. A *P*-value <0.05 denoted the presence of a statistically significant difference.

Results

BALF differential cell count

The BALF components of all groups are shown in Table 1. All patient groups showed significantly higher total cell counts and lower mean percentages of alveolar macrophages than controls. The total cell count of patients with COP was also increased significantly compared with those of IPF and sarcoidosis patients. The mean percentage of lymphocytes in BALF was significantly higher in patients with PAP, NSIP, COP and sarcoidosis than in controls. The mean percentage of eosinophils in BALF was significantly higher in patients with PAP and IPF than in controls and patients with sarcoidosis. There were no significant differences in the mean percentage of neutrophils among the groups.

HAD and HBD levels in BALF and serum

Figure 1A shows the HAD levels in BALF measured by means of RIA in the 72 study subjects. Levels of HAD in BALF of patients with PAP (152 [1–327] pg/ml) were increased significantly compared with controls (0 [0–66] pg/ml) and patients with COP (16.5 [0–57] pg/ml) and sarcoidosis (4.2 [0–161] pg/ml). BALF levels of HAD in patients with IPF (7.7 [0–709] pg/ml) were significantly higher than those in patients with sarcoidosis. BALF levels of HBD-1 and -2 in patients with PAP (905.9 [161.0–1509.8] pg/ml and 56.5 [0–960] pg/ml, respectively) were significantly higher than those in controls (50.2 [14.3–89.7] pg/ml and 0 [0–2.1] pg/ml, respectively) and other lung diseases (Fig. 2A and 3A). Serum levels of HAD in all disease subjects were significantly higher than in controls (Fig. 1B) and there were no significant differences among all patient groups. Serum levels of HBD-1 in patients with IPF, NSIP, COP and sarcoidosis were increased significantly compared with controls, and those with IPF and sarcoidosis were also increased significantly compared with PAP (Fig. 2B). Serum levels of HBD-2 in patients with IPF and sarcoidosis were significantly higher than those in controls (Fig. 3B).

PAP patients have auto-antibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF)[19] and an

Table 1 General characteristics of BALF in patients with interstitial lung diseases.

Patients	*n*	Total cells $\times 10^4$ cells/ml	Cell differential (%)			
			Macrophages	Lymphocytes	Neutrophils	Eosinophils
Control	9	11.0 (2.0–37.0)	90.5 (85.2–96.1)	7.8 (2.6–11.5)	1.0 (0.1–4.7)	0.4 (0–2.3)
PAP	7	37.3 (26.5–93.4)*	40.4 (16.9–69.4)*,‡	40.4 (12.2–79.9)*,‡	4.2 (0.1–20.1)	1.1 (0–33.4)*
IPF	11	32.2 (10.8–72.0)*,†	78.4 (43.5–88.0)*,†	11.1 (2.9–29.9)†	5.6 (2.0–38.1)	3.6 (0–20.4)*
NSIP	15	39.0(20.2–82.0)*	69.3 (26.2–93.4)*	25.9 (3.5–70.1)*,†	2.1 (0.5–41.4)	1.0 (0–6.4)‡
COP	7	42.7 (26.9–148.8)*	41.5 (7.3–80.9)*	31.0 (15.2–92.7)*	3.2 (0–13.4)	3.5 (0–16.5)
Sarcoidosis	23	29.3 (12.0–69.1)*,†	56.5 (7.6–88.4)*,‡	39.1 (11.5–91.5)*,‡	0.7 (0–43.4)	0.3 (0–5.9)‡,§

Data are median (range). PAP: pulmonary alveolar proteinosis; IPF: idiopathic pulmonary fibrosis; NSIP: nonspecific interstitial pneumonia; COP: cryptogenic organizing pneumonia.
*$P<0.05$ versus control.
†$P<0.05$ versus COP.
‡$P<0.05$ versus IPF.
§$P<0.05$ versus PAP.

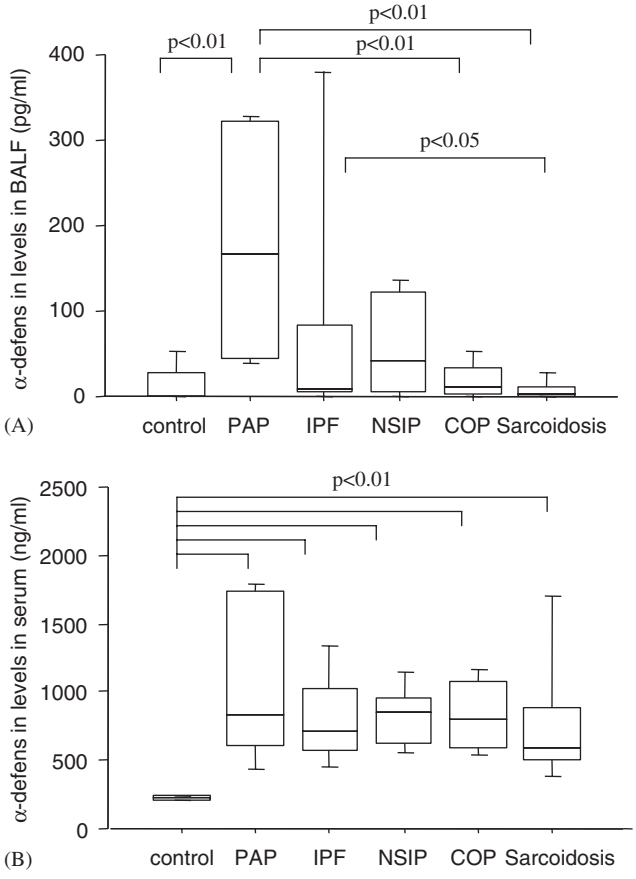

Figure 1 HAD concentrations in BALF (A) and serum (B) of healthy volunteers (control) and patients with interstitial lung diseases. PAP: idiopathic pulmonary proteinosis; IPF: idiopathic pulmonary fibrosis; NSIP: idiopathic nonspecific interstitial pneumonia; COP: cryptogenic organizing pneumonia.

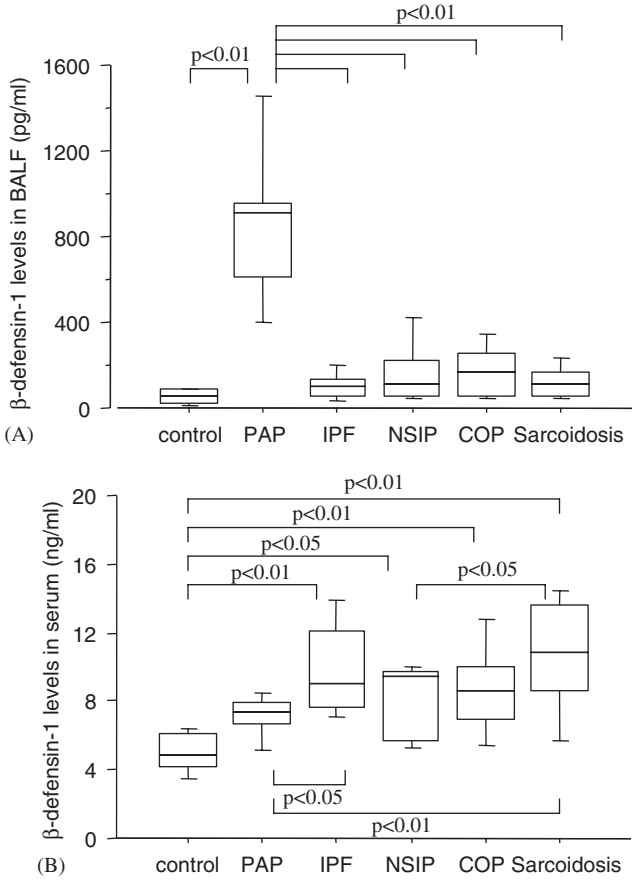

Figure 2 HBD-1 concentrations in BALF (A) and serum (B) of controls and patients with interstitial lung diseases.

elevated level of surfactant protein (SP)-A is found in their serum and BALF.[20] These auto-antibodies and proteins have a possibility of interfering with these immunoassays, so we measured the levels of these defensins after mixing the equal volume of BALF from controls and PAP patients. The total of three PAP patients and healthy volunteers were recruited to this experiment and the BALF mixtures were analyzed. The average levels of HAD and HBD-1, 2 were 682.0, 339.8, and 431.6 pg/ml, respectively, which are almost equal to the mean of defensin levels acquired from independent measurement of BALF from each of the PAP

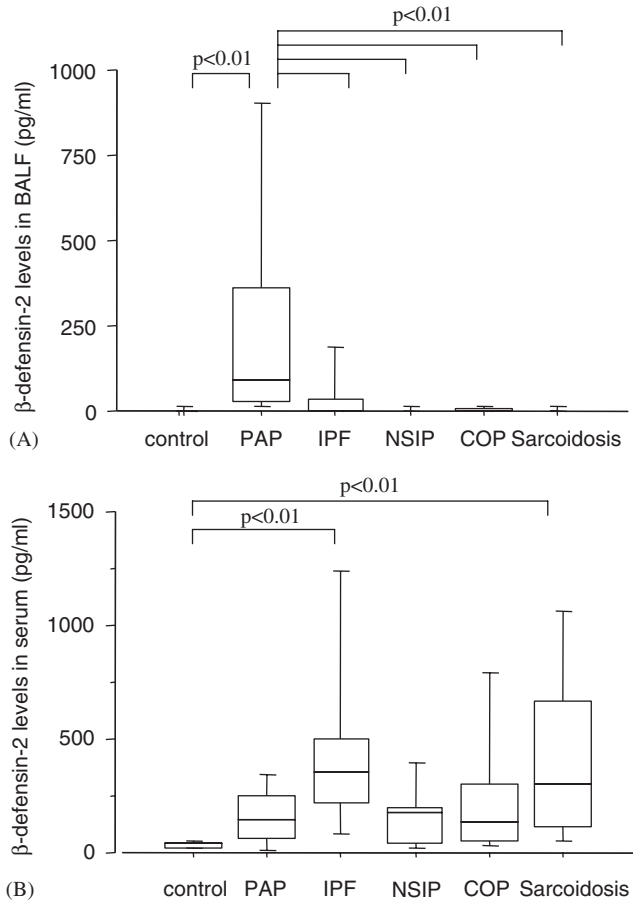

Figure 3 HBD-2 concentrations in BALF (A) and serum (B) of controls and patients with interstitial lung diseases.

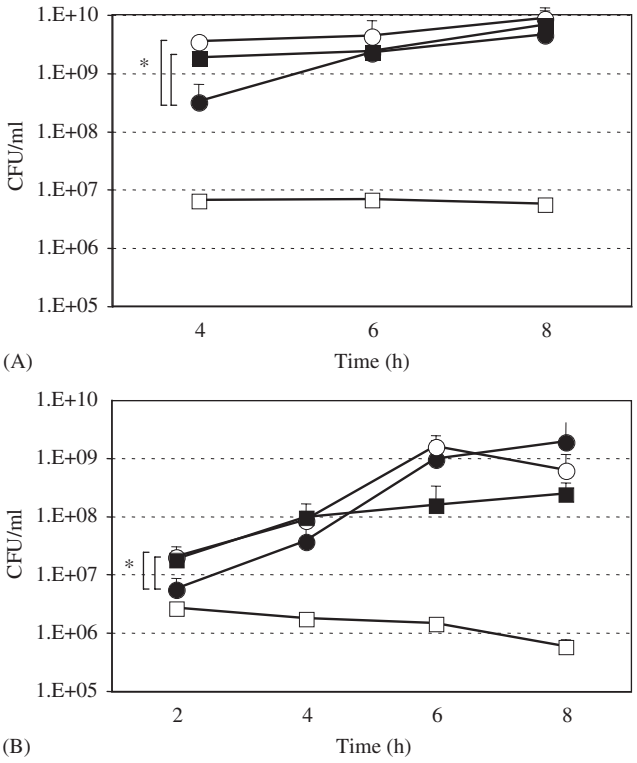

Figure 4 The antimicrobial activities of BALF of patients with PAP, IPF and healthy volunteers against *P. aeruginosa* (A) and *S. aureus* (B). Closed circles, PAP; closed squares, IPF; open circles, healthy volunteers; open squares, saline. $*P<0.05$ compared with each group.

patients and controls. In addition, using these RIA systems, serial diluted solutions of SP-A (kindly provided by Prof. H. Takahashi and Dr. H. Chiba in Third Department of Internal Medicine, Sapporo Medical University School of Medicine) and rat anti-human GM-CSF antibody (Beckman Coulter, Inc., Fullerton, CA) (0–12.8 μg/ml) were measured the defensin levels and the levels of these defensins in all samples were lower than detection limits. These indicate that these auto-antibodies and SP-A in PAP patients might not interfere with these RIA.

Antimicrobial activity of BALF

We performed a colony count assay using the BALF obtained from five healthy controls, six patients with PAP and six with IPF. The BALF from patients with PAP inhibited bacterial growth of *P. aeruginosa* (4-h incubation) and *S. aureus* (2-h incubation) compared with the BALF from healthy controls and patients with IPF (Fig. 4).

Discussion

The major finding of the present study was that there was a distinct profile of serum and BALF levels of HAD and HBD among different interstitial lung diseases. In addition, there were no significant differences in the serum levels of HBD-1 and -2 between PAP patients and controls, while the BALF of patients with PAP had extremely high levels of these defensins and also showed antimicrobial activity against *P. aeruginosa* and *S. aureus*.

PAP is a rare auto-immune lung disease characterized by abnormal surfactant accumulation within alveolar macrophages, and circulating auto-antibodies against GM-CSF resulting in functional GM-CSF deficiency.[19] Surfactant lipids and proteins are synthesized, stored, and secreted into the alveoli by alveolar type II epithelial cells.[19] We initially hypothesized that HBD levels might be elevated in patients with PAP because HBD-2 was detected mainly in alveolar type II cells in the lung by our previous immunohistochemical study.[7] In the present study, the levels of these defensins, especially HBD, in BALF obtained from PAP patients were extremely high and increased significantly compared with controls and patients with other interstitial lung diseases. In addition, the BALF levels of HBD-1 and -2 in PAP were also higher than previously reported levels in diffuse panbronchiolitis (HBD-1 264.1 ± 61.0 pg/ml and HBD-2 71.5 ± 28.7 pg/ml),[7] which is a progressive disease characterized by frequent episodes of superimposed infection by *P. aeruginosa*. An important feature of PAP is the associated susceptibility to pulmonary infections, sometimes with opportunistic organisms. Primary and cultured alveolar macrophages from mice with targeted disruption of the gene encoding GM-CSF have defects in cellular adhesion, expression of pathogen-recognition receptors, phagocytosis, superoxide production, microbial killing and secretion of

proinflammatory cytokines.[19] This macrophage dysfunction may account for the susceptibility to infections in this disease. In the present study, extremely high levels of both HAD and HBD in BALF were observed in PAP. Although alveolar macrophages are known to be cell source of these defensins,[21] the elevation of defensins in BALF of PAP patients may be due to reduced clearance by alveolar macrophage dysfunction. In this study, an antimicrobial activity against *P. aeruginosa* and *S. aureus* was also observed in the BALF of patients with PAP, but not IPF and controls. Since defensins are the most prominent antimicrobial factors in airway surface fluid,[22] antimicrobial peptides including defensins in the lung may contribute to prevention of bacterial infection in the lung of patients with PAP. In this context, SP-A, has also bactericidal activity,[23] is known to be elevated in BALF of PAP patients,[20] so SP-A may also contribute to this BALF activity.

Meanwhile, the serum levels of HBD in PAP patients were similar to control subjects. On light-microscopic examination, the architecture of the PAP lung parenchyma is preserved and the walls of transitional airways and alveoli are usually normal.[19] Therefore, the HBD secreted into the airspace may not influx into the bloodstream in PAP. In this context, the levels of KL-6, a mucin-like high-molecular weight glycoprotein, were found to be elevated in patients with various interstitial lung diseases, especially PAP.[24] In PAP, the origin of the elevated KL-6 is suggested to be proliferating type II pneumocytes, like HBD-2. Markedly high levels of KL-6 are found in serum as well as in BALF in PAP patients, suggesting that KL-6 produced abundantly by type II pneumocytes leaks into the bloodstream from the alveoli.[24] Defensins are suggested to leak into the circulation from the alveoli more easily than KL-6 because they are small peptides with a molecular mass of 3.5–5 kDa. Further investigations are required into the alveolar-capillary barrier and the clearance of these molecules in this disease.

Levels of HAD, mainly localized in neutrophils, were also increased significantly in BALF of patients with PAP compared with controls and patients with COP and sarcoidosis. Cell analysis of BALF from PAP patients revealed a significantly increased percentage of lymphocytes, but not neutrophils (Table 1), consistent with a previous report.[25] However, the absolute number of neutrophils in BALF was increased significantly due to a significantly higher total cell count in BALF from PAP patients. Therefore, we suggest that the origin of HAD in the PAP lung is neutrophils in the airway lumen, and that the elevation of HAD in BALF of PAP patients may be due to reduced clearance, like other surfactant lipids and proteins.[19] Serum levels of HAD in all patient groups were significantly higher than those in controls. HAD are secreted from neutrophil precursor cells in the bone marrow into the plasma as pro-defensins, where they make up 25% of the plasma HAD molecules in normal subjects.[15] In our previous studies, the rise of plasma HAD levels in patients with infectious lung diseases was mainly derived from activated neutrophil precursor cells in the bone marrow.[6,26] Therefore, we suggest that mediators released from inflammatory cells and epithelial cells in the lung of interstitial lung diseases may also directly stimulate the bone marrow to enhance the release of HAD from precursor cells in the bone marrow into the circulation, as in infectious lung diseases.

Serum levels of HBD-2 in patients with IPF and sarcoidosis were significantly higher than those in controls. In addition, serum levels of HBD-1 in these patients were significantly higher than those in controls and patients with PAP, while there were no significant differences in BALF HBD levels between controls and IPF and sarcoidosis. In IPF patients, we previously reported that levels of HAD in plasma were significantly higher compared with control subjects and reflected the clinical course and pulmonary function tests.[10] These results suggest that serum but not BALF levels of HBD as well as HAD may be associated with the pathogenesis of sarcoidosis and IPF, similar to infectious lung diseases.[5,7]

In summary, we have demonstrated the presence of differential profiles of serum and BALF defensins in different interstitial lung diseases. Our results suggest that patterns of defensin elevation in BALF may be helpful in distinguishing PAP from other lung diseases and that these antimicrobial peptides in airway may contribute to prevention of bacterial infection in PAP.

Acknowledgments

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